WD-40 repeat protein SG2NA has multiple splice variants with tissue restricted and growth responsive properties

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SG2NA is a member of the striatin family of WD-40 repeat proteins with potential scaffolding functions. It was originally identified as a tumor antigen with increased expression during S to G2 phase of cell cycle. We report here that mouse SG2NA has at least five novel splice variants of which two are devoid of the carboxyl terminal WD-40 repeats. The variants of SG2NA are generated by alternative splicing at the exon 7–9 regions and differ in their expression profiles in various tissues tested. While the 83, 78, 38 and 35 kDa variants are present in both brain and heart, the 87 kDa form is brain specific. Also, the expression of 35 kDa variant is more in neonatal than in adult tissues. Western analysis suggests that the SG2NA isoforms differentially respond to growth stimuli. Upon serum stimulation, while the 35 kDa variant is increased, the 78 kDa form is diminished. Splicing variation of SG2NA is conserved in metazoan evolution. In embryonic chicken there are at least four variants of which one is present in brain but absent in heart. Taken together, splicing variation of SG2NA might have some critical roles in differentiation and maturation in metazoan cells.

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1. Introduction

SG2NA is a member of the striatin sub-family of WD-40 repeat proteins (Benoist et al., 2006; Castets et al., 1996). The prototype member of the family was first characterized from rat brain synaptosomes and later found in abundance in the striatum, hence named striatin (Castets et al., 1996; Salin et al., 1998). SG2NA was originally identified as a nuclear autoantigen whose expression is augmented during S to G2 phase of cell cycle while its structural and functional relatedness to striatin was established thereafter (Muro et al., 1995; Castets et al., 2000). In addition to the WD-40 repeats present in their carboxyl terminus, striatin family members are also characterized by a caveolin binding motif, a coiled–coiled structure, and a calmodulin binding domain located in the amino terminus in the same order (Benoist et al., 2006; Castets et al., 2000; Gaillard et al., 2001). The coiled-coiled motif of striatin is involved in oligomerization and is required for targeting it to the dendritic spines (Gaillard et al., 2006). In agreement with the proposed scaffolding functions (as WD-40 repeat proteins), striatin and SG2NA have been shown to interact with phoerin and Protein Phosphatase 2A (PP2A), thereby attributing them to vesicular trafficking and cell signaling respectively (Baillat et al., 2001; Moreno et al., 2000). In addition, striatin has also been reported to interact with estrogen receptor, thus contributing towards non-genomic steroid signaling (Lu et al., 2004). Members of the striatin family are conserved in metazoan evolution and are absent in plants and prokaryotes (Benoist et al., 2006).

Noticeably, Drosophila has only one striatin homologue i.e., CKA, which acts as a platform for organizing the components of JNK signaling and the transcription factor AP-1, indicating a common functional ancestry between the three members of the family (Chen et al., 2002). Taken together, SG2NA and other members of the striatin family presumably perform certain specialized functions in metazoan organisms that are yet to be deciphered.

WD-40 proteins are characterized by the presence of multiple repeats of ~40 minimally conserved amino acids with a pair of glycine–histidine residues (GH) at the amino terminus and a pair of tryptophan–aspartic acid (hence named WD) residues at the carboxyl terminus (Neer et al., 1994). The WD-40 repeats occur in tandem and their numbers generally vary from 4 to 8, although proteins with as low as two and as many as sixteen repeats have also been reported (van Nocker and Ludwig, 2003; Saeki et al., 2006; Li and Roberts, 2001). The WD-40 repeat sequences are highly conserved in evolution.

Abbreviations: BLAST, Basic Local Alignment Search Tool; CKA, Connector of Kinase to AP-1; EST, Expressed Sequence Tag; JNK, Jun N-Terminal Kinase; siRNA, small interfering RNA; Strn, Striatin; Strn3, Striatin3/SG2NA.

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and a large number of cognate proteins have been found in mammals, plants, Drosophila, C. elegans, yeasts, and in prokaryotes (van Noeker and Ludwig, 2003; Saeki et al., 2006; Li and Roberts, 2001). Members of the WD-40 repeat family have been attributed to biological functions as diverse as RNA transcription and processing (de la Cruz et al., 2005), vesicular trafficking (Fritzius et al., 2007), cytoskeletal assembly (Pollmann et al., 2006), cell signaling (Fritzius et al., 2006), cell cycle (Yoon et al., 2004), and cell death (Ogawa et al., 2003). It is believed that rather than having a catalytic function, WD-40 domains provide a scaffolding platform for interaction between different proteins, thereby explaining its functional diversity (Li and Roberts, 2001).

Taken together, although two independent lines of research over the past decade have identified SG2NA as a WD-40 repeat protein with diverse roles in cell cycle regulation (Muro et al., 1995), cell signaling (Moreno et al., 2000), vesicular trafficking (Gaillard et al., 2006), and in gene regulation (Zhu et al., 2001), its exact function in metazoan biology is unknown (Benoist et al., 2006). In the present study, we, for the first time report that metazoan SG2NA exists in multiple isoforms generated out of alternative splicing. We also demonstrate that the splice variants of SG2NA are differentially expressed in various tissues and their expression profile changes with the state of differentiation and the proliferative potential of the cognate cell type.

2. Materials and methods

2.1. Reagents

All restriction enzymes and modifying enzymes were from MBI Fermentas Inc., USA. RNase inhibitor was from New England Biolabs, USA and fetal bovine serum was from Hyclone, USA. All other reagents including oligonucleotide primers were from Sigma-Aldrich, USA.

SG2NA siRNA (sc-37648) and Control siRNA (sc-37007) were from Santacruz Biotechnology, USA.

2.2. Primers

P1: 5'-GGTTGGAATCTCTTAAGGC-3', P2: 5'-GGCAATTATTTGG-GACGGC-3', P3: 5'-GACTCTTTAAGCAGGA-3', P4: 5'-CTAAATTTAAC- CATGGC-3', P5: 5'-TACCTCTAAAAGCCAACTG-3' and P6: 5'- AATTCACTGGTGAGCGAT-3'.

2.3. Cell lines

H9c2, C6, HepG2, Neuro2a and NIH3T3 cell lines were obtained from National Centre for Cell Sciences, Pune, India. Cells were maintained as monolayer cultures (till ~20 passages) in DMEM, high glucose, supplemented with 10% fetal bovine serum or 10% calf serum (only for NIH3T3 cells), 90 U Penicillin/Streptomycin and 5 μg/ml amphotericin B in a humidified incubator containing 5% CO2 at 37 °C.

2.4. Nucleotide/peptide sequence analysis

ESTs corresponding to variants of mouse SG2NA were retrieved from NCBI database using BLAST tool using the published nucleotide sequence of SG2NA as the input. The exon–intron boundaries of the SG2NA transcript were determined through the “Ensembl” Genome Browser (www.ensembl.org). Alignments of peptide sequences were done using “MultAlin” program (bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html). Open reading frame of cDNA sequences were translated using “ExPASy” (http://au.expasy.org/).

2.5. Bacterial expression of SG2NA

A recombinant plasmid pGEX4T3MS, harboring the GST coding sequence in frame with that of the 35 kDa SG2NA (originally procured as IMAGE Clone: 2101786 from Open Biosystems, USA) was transformed into E. coli BL-21DE3 cells. Primary culture was grown from isolated colonies in LB broth with ampicillin (100 μg/ml) at 37 °C, 220 rpm overnight. Secondary culture in fresh LB-ampicillin (100 μg/ml) medium (37 °C) at OD600=0.4 was induced with 1 mM IPTG and grown further for 4 h. Bacterial lysates were prepared in Laemmli’s Buffer [60 mM Tris.HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 0.002% (w/v) bromophenol blue] were subjected to 10% SDS-PAGE followed by staining with Coomassie Brilliant blue [CBB, 0.1% (w/v) in 45% (v/v) methanol and 10% (v/v) acetic acid].

2.6. Purification of recombinant protein

Recombinant GST-35 kDa SG2NA (expressed from pGEX4T3MS) was purified from the insoluble fraction of bacterial lysate using ionic detergent Sarkosyl, essentially as described by Frangioni and Neel (1993). IPTG induced bacterial culture (100 ml) was centrifuged at 5000 rpm for 10 min and the bacterial cell pellet thus obtained was washed and resuspended in 9 ml ice cold STE [10 mM Tris.HCl (pH7.5), 100 mM NaCl, 1 mM EDTA]. Lysozyme was added to a final concentration of 100 μg/ml followed by incubation at 30 °C for 15 min. After lysis, DTT (5 mM), protease inhibitors (1X, bacterial cocktail), 1 mM PMSF) and sarkosyl (to a final concentration of 1.5%) were added. The lysate was then mixed thoroughly by vortexing, sonicated and centrifuged at 10,000 rpm, 4 °C, for 10 min. Finally, Triton X 100 was added to the supernatant to a final concentration of 2% and swollen GSH beads, equilibrated with PBS. Protease inhibitors were added to the mixture and kept for overnight binding at 4 °C. Protein bound beads were collected by pulse centrifugation, rinsed in 1X wash buffer [10 mM Tris HCl (pH 7.6), 400 mM NaCl, 1 mM EDTA] and protease inhibitor cocktail (1X, bacterial) and 1 mM PMSF] and denatured in Laemmli’s Buffer followed by 8% SDS-PAGE and staining with CBB.

2.7. Mass spectrometric analysis

Mass spectroscopic analysis of peptide fragments obtained by Tryptic digestion of purified recombinant GST-35 kDa SG2NA (excised from CBB stained SDS-polyacrylamide gel) was done using Autoflex II MALDI-TOF/TOF, Bruker Daltonics, Germany.

2.8. Antibodies

Four hundred micromgrams of purified, recombinant GST-35 kDa SG2NA was resolved on 8% SDS-PAGE and the induced protein band (60 kDa, as identified by CBB staining of flaming reference lanes loaded with same samples) was excised out and crushed. The crushed gel slice containing the antigen was mixed with complete Freund’s adjuvant, and injected subcutaneously into 3 month old male rabbit. The first booster dose of 200 μg protein (gel immobilized) with incomplete Freund’s adjuvant was administered after 4 weeks of priming followed by another booster immunization after 2 weeks. Antiserum were collected 10 days after each injection. Goat polyclonal antibodies against SG2NA (sc-16855, Santacruz Biotechnology Inc., USA) was also used for some experiments as indicated in respective figure legends. While the antibody we had raised was efficient in recognizing all the predicted isoforms of SG2NA, the commercial antibody was more efficient in detecting the larger (78–87 kDa) isoforms and poor in detecting the smaller (35 kDa) isoform.
2.9. Preparation of tissue extracts

Mouse tissues were snap frozen in liquid nitrogen immediately after dissection and homogenized in ice-cold RIPA buffer (50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X 100, 1% (w/v) Sodium Deoxycholate, 0.1% (w/v) SDS, 1 mM PMSF, 1 mM DTT and protease inhibitors cocktail (1X, mammalian, Sigma)) in an all glass Dounce homogenizer. The homogenates were separated into pellet and supernatant fractions by centrifugation at 15,000 g for 1 h at 4 °C. Subcellular fractionation of tissue extracts were prepared as described by Pipkin et al. (2003).

2.10. Ammonium sulfate fractionation of tissue extracts

Supernatant fractions (1,50,000 g, 10 mg/ml) of tissue extracts prepared in RIPA buffer were subjected to ammonium sulfate (50%) fractionation on ice. The precipitated proteins were then collected by centrifugation at 13,000 g, for 20 min, 4 °C, desalted in Sephadex G-25 column; followed by Western analysis.

2.11. Preparation of extracts from cultured cells

Cells were grown on 90 mm culture dishes up to 60–70% confluence, scraped in ice cold PBS and collected by centrifugation at 3000 rpm, 4 °C for 10 min. Cell pellets were lysed in RIPA buffer containing 1 mM PMSF; 1 mM DTT and protease inhibitors cocktail (1X, mammalian, Sigma) followed by centrifugation at 13,000 rpm. The supernatant was used for Western analyses.

2.12. Reverse transcription-PCR

Total RNA from tissue was isolated using TRI Reagent, Ambion. First strand cDNA synthesis was done using Revert-Aid M-MuLV Reverse Transcriptase, MBI Fermentas Inc., USA. Polymerase chain reaction was performed with cDNA template using appropriate primer pair.
2.13. Western blot

Concentrations of proteins were estimated by modified Bradford’s method. Equal amounts of test proteins were prepared in SDS sample buffer (Laemmli’s buffer), resolved by 10% SDS-PAGE and transferred to charged PVDF membrane. Membranes were blocked in 3% BSA in TBST [10 mM Tris.HCl (pH 7.4), 150 mM NaCl, 0.05% tween-20], immuno-blotted with the 0.1 μg/ml.antiseria in TBST followed by incubation with HRP conjugated anti-rabbit/goat IgG (Santacruz Biotechnology, Inc, CA, USA). Immunocomplexes were visualized with enhanced chemilumi-nescence detection reagents, Sigma Aldrich, USA.

2.14. siRNA transfection

SG2NA siRNA (80 nM) or control siRNA (20 nM) was transfected into cells at 50% confluence using Superfect Transfection Reagent, Qiagen as per manufacturer’s protocol.

2.15. Validation of data

Each western blot was repeated thrice and a representative blot has been shown.

3. Results

3.1. SG2NA has a 35 kDa variant devoid of WD40 repeats

We recently had developed a molecular approach for isolating a repertoire of cis-regulatory DNA sequences involved in embryonic chick heart development (Sindhu et al., 2004). Thereafter, a number of novel sequences were used as probes for screening a 72 hour embryonic chick heart cDNA expression library and amongst the isolates was a partial cDNA clone of chicken SG2NA (unpublished results). The DNA binding and transactivation potential of SG2NA have been reported earlier (Zhu et al., 2001; Landberg and Tan, 1994). Although SG2NA is expressed in heart (and several other tissues) at a higher level, its function in cardiovascular biology has not been investigated (Castets et al., 2000). We thus intended to explore its function of SG2NA in heart development and procured the mouse SG2NA cDNA from Open Biosystems, USA (IMAGE: 2101786). The identity of the cDNA was initially confirmed from the source by sequencing the 5’ end and its size was ~3 kb as expected. However, upon complete sequencing, the cDNA turned out to be a novel form of SG2NA, having a 35 kDa open reading frame wherein the first 282 aa are identical to that of the reported 87 kDa form (Castets et al., 1996; Castets et al., 2000), followed by an extra stretch of 19 amino acids and then a stop codon (Fig. 1A, sequence submitted to the NCBI database under Acc. No. EF685152). Thus, the 35 kDa variant of SG2NA is devoid of the 514 aa encompassing the WD-40 repeats present at the carboxyl terminus of 87 kDa SG2NA. The cDNA was then expressed in E. coli as a 60 kDa GST fusion protein (Fig. 1B) and its identity was established by mass spectrometric analysis (Fig. 1C).

3.2. SG2NA has other variants that are differentially expressed in heart and brain

We thereafter searched the mouse EST database for other possible variants of SG2NA and identified at least four more isoforms, apparently arising out of alternative splicing at exon 7–9 region (Fig. 2A, upper panel). Noticeably, EST analysis did not show the presence of 35 kDa variant we had identified, thus raising the possibility that more variants might exist. We, therefore, designed two primers from the exon 5 and 11 (P1 and P2, see Materials and methods for details) of mouse sg2nastrn3 gene and analyzed the total RNA from neonatal mouse heart and brain for its expression profile by RT-PCR. The PCR products were cloned, sequenced, matched with the EST database (submitted to the NCBI under accession nos. EF685153, EF685154 and EF685155) and the corresponding protein sequences were derived as 87, 83, 78, 38 and 35 kDa’s (Fig. 2B and C). We thereby detected five variants of SG2NA expressing in brain (Fig. 2D, left and the middle panels). Further, although the RT-PCR analysis was semi-quantitative, we consistently observed that the relative efficiency of amplification of the transcript corresponding to 87 kDa was more than others (Fig. 2D, left panel). When the profile of expression was examined in heart, it was similar to that in brain, except that the 87 kDa form was absent (Fig. 2D, left panel).

As in case of heart, the relative efficiency of amplification of the transcript corresponding to the 78 kDa was consistently high as compared to others, we also confirmed the absence of 87 kDa variant by Southern analysis of the RT-PCR products (Fig. 2D, middle panel). Furthermore, since the amplicon corresponding to 35 kDa and 38 kDa were barely detectable in heart (Fig. 2D, left panel), we also performed PCR analysis of those transcripts by using sequence specific primers, P3/ P4 (see Materials and methods in details) and confirmed their presence (Fig. 2D, right panel). Noticeably, the 82 kDa form predicted from EST analysis was absent in both heart and brain and thus might be expressing in other tissues.

3.3. Post-natal modulation of SG2NA variants

RT-PCR analysis suggested that the variants of SG2NA are differentially expressed in neonatal mouse heart and brain. We thus explored their expression during post natal development. Polyclonal antiserum was raised against the bacterially expressed 35 kDa isoform (Fig. 1A). Since all the predicted variants of SG2NA have a common amino terminus (–282 aa, Fig. 2C), the antiserum recognized all the potential variants of SG2NA as identified by their predicted molecular weights. However, we experienced that the antiserum also cross-reacted with number of other proteins (as shown below in Fig. 3C). Further, while this work was in progression, antibody against SG2NA became commercially available (sc-16855, affinity purified, SantaCruz Biotechnology, USA) but that also showed similar cross reactivity. Therefore, for Western analysis of tissue extracts, we partially purified the SG2NA proteins by ammonium sulfate (50%) fractionation. Our preliminary data also suggested that while the larger variants of SG2NA are primarily cytosolic, the 35 kDa form is membrane associated (1,50,000 g supernatant and pellets respectively). Western analysis of the ammonium sulfate fractionated cytosolic extracts from post natal-mouse brain showed that the expression of 87 and 83 kDa isoforms gradually diminished with increasing age, while that of the 78 kDa form slightly increased (Fig. 3A, right panel). We also observed that the 35/38 kDa variant was primarily membrane associated while its expression was higher in day 0 till day 7 and diminished thereafter (one and two months, Fig. 3A, right panel). At this stage, it was not possible to ascertain whether the immune reactive band represented 35 kDa or 38 kDa or both, as they are likely to comigrate in SDS-PAGE (RT-PCR analysis showed the presence of both 35 and 38 kDa in newborn heart and brain). To further validate the modulation (both expression and sub-cellular distribution) of the 87, 83 and 78 kDa variants, heart and brain extracts from newborn (one day) and adult (one month) mouse were separated into the nuclear/cell debris (3000 g pellet), membrane (1,000 g pellet) and cytosolic fractions (post 1,000 g supernatant) and simultaneously Western analyzed. As shown in Fig. 3B, the 87 kDa isoform was absent in heart but present in brain as expected (from the RT-PCR analysis). Also, both the 83 and 87 kDa forms were more in neonatal brain. Notably, all the three larger isoforms (87, 83 and 78 kDa) were present in both membrane and cytosolic fractions, whenever they had expressed. However, since the membrane fractions were highly enriched while the cytosolic fractions were unfraccionated, their relative abundance in those fractions is not necessarily a reflection of their natural distribution between the membrane and cytosol. Finally, to validate the identity of each of those SG2NA variants, selective knock-down
Each band of interest is indicated by a number and the key is given below as a table. Obtained from another independent experiment was done using PCR products amplified from neonatal mouse heart (MHRT) and brain (MBRT) using primers P1 and P2 to the 87 kDa position remained intact. It is not clear yet whether NIH3T3 cells express 87 kDa SG2NA or not (it does not express in mouse heart but expresses in brain). We thus used H9c2 rat cardiac myoblast cells to confirm the identity of the 87 kDa form (RT-PCR analysis suggests that H9c2 cells express 87 kDa, data not shown). As shown in Fig. 3C (right panel); in H9c2 cells the 87 kDa band was silenced by siRNA. Interestingly, another band of ~47 kDa was also silenced in both H9c2 and NIH3T3 cells which could probably represent SG2Na mRNA isoform recently reported in rat (DQ473607).

3.4. 35 kDa SG2NA is abundantly expressed in immortalized cells

In view of our observation that newborn mouse tissues have higher levels of the 35/38 kDa SG2NA than their adult counterparts (and as per the original report that SG2NA is a cell cycle associated protein), we explored the possibility that the 35/38 kDa variant is a characteristic of proliferating and undifferentiated cells. Accordingly, we tested a number of cell lines viz., H9c2 (rat cardiac myoblast), HepG2 (human hepatocellular carcinoma), C6 (rat glioma), and Neuro 2a (mouse neuroblastoma) and consistently observed that all these cells had a higher level of expression of the 35/38 kDa form (Fig. 4A). Noticeably, C6 cells had an immunoreactive band at ~47 kDa that was absent in H9c2 myoblasts, although both are of rat origin. Taken together, these results suggest that the ~35 kDa SG2NA is expressed at a higher level in immortalized rat and mouse cells.

3.5. Some variants of SG2NA are growth responsive

Since SG2NA is a cell cycle regulated protein, we checked if the other variants are also associated with cell growth/proliferation. Actively growing H9c2 cells were kept in serum free medium for 12 h, 24 h and 36 h and the levels of the 87, 83, 78 kDa forms of SG2NA were checked by

Fig. 2. (A) Database analysis demonstrating the existence of multiple splice variants of mouse SG2NA: Mouse ESTs homologous to the nucleotide position 870 to 1587 of known isoform of SG2NA (NM052973) are shown by reproduction of the NCBI BLAST analysis. The nucleotide position of the input SG2NA sequence is shown on top and homologous output EST sequences are shown below. The hatched stretch in each band represents a region of the EST sequence that is either spliced out or is differentially spliced in (and thus non-homologous to the input sequence). The predicted molecular weight of each variant is marked on left that was con

Fig. 3. (A) Expression of SG2NA during post-natal development: (A: Left panel) Extracts were prepared from mouse brains of different age groups and the 1,50,000 g supernatant was further enriched by ammonium sulfate precipitation (50%, see Materials and methods for details). Two hundred micromolar of each sample was Western analyzed for the 87, 78 kDa bands of SG2NA. Equal protein loading was ensured by staining the PVDF membrane with Coomassie Brilliant Blue as shown in the lower panel. (A: Upper right Panel): Seventy five microgram of ammonium sulfate fractionated mouse brain tisucal extracts were Western analyzed for the 35/38 kDa isoform of SG2NA. Equal amount of the pellet fraction from day 0 brain extract was included as positive control. Protein loading, especially in the first four lanes where 35 kDa was absent is confirmed by Coomassie Brilliant Blue staining of the membrane. In addition, a non specific immunoreactive band of higher molecular weight, further ensuring the integrity of proteins, is also shown wherein the 35/38 kDa band is absent. (A: Lower Right Panel): Western analysis of the 1,50,000 g pellet fractions of mouse brain extracts for the 35/38 kDa SG2NA. The membrane stained with Coomassie Brilliant Blue is shown below for ensuring equal loading. (B) Nuclear (3000 g pellet, 200 g) membrane (1,00,000 g pellet, 50g) and the cytosolic (post 1,00,000 g supernatant, 200 g) fractions prepared from neonatal (1 day) and adult (one month) mouse heart and brain were resolved on 8% SDS-PAGE and the regions corresponding to the high molecular weight isoforms of SG2NA (78-87 kDa) were immunoblotted with the polyclonal antiserum. (C) Identification of specific isoforms of SG2NA using siRNA. NIH3T3 (Left Panel) and H9c2 (Right Panel) cells were transfected with siRNA specific to mSG2NA or randomized control. Extracts were prepared and Western analysis was done using polyclonal antisera. The specific isoforms silenced by siRNA are indicated by arrows and corresponding molecular weights are also shown.

experiments were done by transfecting mouse SG2NA siRNA (sc-37648) into NIH3T3 fibroblasts followed by Western analysis. As shown in Fig. 3C, left panel; transfection of siRNA, distinctly silenced the 35 kDa and the 78 kDa isoforms. However, the band corresponding to the 87 kDa fraction followed intact. It is not clear yet whether NIH3T3 cells express 87 kDa SG2NA or not (it does not express in mouse heart but expresses in brain). We thus used H9c2 rat cardiac myoblast cells to confirm the identity of the 87 kDa form (RT-PCR analysis suggests that H9c2 cells express 87 kDa, data not shown).

Noticeably, C6 cells had an immunoreactive band at ~47 kDa that was absent in H9c2 myoblasts, although both are of rat origin. Taken together, these results suggest that the ~35 kDa SG2NA is expressed at a higher level in immortalized rat and mouse cells.
immunoblotting. As shown in Fig. 4B, while both 87 and 78 kDa SG2NA showed a decrease in expression till 36 h, the 83 kDa form showed slight increase upon serum deprivation. Noticeably, unlike in mouse heart tissue that expresses the 78 kDa variant only, H9c2 myoblasts showed moderate expression of both 87 and 78 kDaAs and only a barely detectable level of the 83 kDa form. Currently we do not know whether this difference is due to immortalization of H9c2 cells or it is a species associated phenomenon. When those quiescent cells were subjected to serum (10%) treatment, both 87 and 78 kDa forms were re-induced only till 24 h while the 78 kDa form was downregulated thereafter (till 36 h, Fig. 4B). Also, the 83 kDa form was obliterated upon serum treatment (Fig. 4B). Upon testing the effect of serum deprivation/stimulation on the 35 kDa variant, we observed that it is more in growing cells vis-à-vis quiescent cells (Fig. 4C). Taken together, it appears that various forms of SG2NA differentially respond to serum stimulation/deprivation.

3.6. Splicing variation of SG2NA is conserved in other metazoan species

Proteins of striatin family are found in mammals (mouse, rat and human), in lower vertebrates (D. rerio, X. laevis and G. gallus), and in invertebrates (D. melanogaster and C. elegans) but not in prokaryotes, yeasts and plants (Benoist et al., 2006). In contrast, WD-40 repeat superfamily (of which striatin is a member) is conserved throughout evolution (van Nocker and Ludwig, 2003). It is, thus, likely that striatin proteins might have certain specialized functions in metazoan organisms that got further diversified by alternative splicing. To assess such possibility, we performed a phylogram analysis, which showed that SG2NA sequences are orthologs originating from a common ancestor (Fig. 5A). We then argued that, monitoring the splice variations of SG2NA in other species might give further insights into its importance in metazoan biology. We thus identified the chicken SG2NA sequences and performed a phylogram analysis as shown in Fig. 5B. The phylogram was constructed using ClustalW alignment of the 7 SG2NA sequences from different species using the TreeTop (http://www.genebee.msu.su/services/phtree_reduced.html) program. The length of branches (X-axis) in the rectangular phylogram represent the relative distances among those protein sequences calculated using BLOSUM62 substitution matrix. Numbers beside the branching nodes are the bootstraps values. (B) Splicing pattern of SG2NA in embryonic chicken tissues (left panel): Total RNA (2.5 μg) isolated from 12 day embryonic chicken tissues (Heart; Brain; Skeletal muscle and Liver) were subjected to RT-PCR analysis using primers P5/P6. The PCR products were resolved on 1.5% agarose gel followed by Southern analysis using α32P random labeled probe derived from the clone, IMAGE: 2101786. Right panel: The RT-PCR products were sequenced; the peptide sequences of the corresponding variants were derived and compared. The varying regions are shown in black while the adjoining homologous regions are shown in red (C) Schematic representation of various predicted forms of chicken SG2NA: The conserved (amino and the carboxyl termini) as well as the non conserved predicted variants of cSG2NA are identified respectively by various shades/patterns.
sg2na gene and analyzed its EST profile. We observed that, unlike in mouse, where multiple splicing occurs only in exon 7–9 regions; in chicken, differential splicing occurs at two locations viz., at exon 8–9 and at exon 12 (one each of the respective variants was found in the NCBI EST database; results not shown). Accordingly, we designed two primer pairs spanning exon 8–9 and exon 12 and analyzed the total RNA isolated from embryonic chick (12d) heart, brain, skeletal muscle and liver by RT-PCR. Interestingly, contrary to the EST analysis, we observed that there are at least four variants arising out of splicing at exon 8–9 (Fig. 5B, left panel). The PCR products were then cloned, sequenced (submitted to the NCBI database under accession No. EF685157, EF685158, EF685159, and EF685156) and the corresponding protein sequences were reconstituted (Fig. 5B, right panel). Such analysis revealed that while the largest variant (V1) is present only in brain, the other three variants i.e., V2, V3 and V4 were uniformly present in brain, liver, heart and skeletal muscle, thereby reiterating the pattern found in mouse (Fig. 5C). However, similar analysis with exon 12 region failed to detect any variants other than the full length one (data not shown); leaving the possibility that splicing at exon 12 might be the characteristics of tissues/developmental stages other than those we had tested.

4. Discussion

With the advent of tools of genomics and proteomics, it now appears that organismal complexity is not achieved by the linear increase in gene number but by the intricacy in its regulation. Furthermore, contrary to the initial perception that gene expression is primarily controlled at the level of transcription, recent data suggest that mRNA metabolism viz., stability, processing, transport, and translation significantly add towards regulation of gene function, especially in metazoan organisms (Blencowe and Khanna, 2007).

Alternative splicing has long been known as one amongst a number of post transcriptional processes in eukaryotes. However, its potential exploits in diversifying cellular proteome, have only lately been appreciated (Blaustein et al., 2007). It now appears that about three fourth of human protein coding genes undergo alternative splicing and certain genes may have as many as >10,000 splice variants (Blaustein et al., 2007; Zipursky et al., 2006). Taken together, alternative splicing appears to be the most versatile tool for regulating gene function in higher eukaryotes, including plants.

The present study was initiated for investigating the role of SG2NA in cardiovascular biology. Being a WD-40 repeat protein with caveolin binding function, it was anticipated that SG2NA might have a role in mediating certain pathophysiological signals from the extracellular milieu to the nucleus. However, in the process we identified a novel 35 kDa variant of mouse SG2NA that is devoid of the WD-40 repeats, might be a potential candidate for a constitutively activator of its transactivation function or a dominant negative inhibitor of its transactivation function (Benoist et al., 2006). Nevertheless, such changes in the protein-coding region might have profound effects like altered intracellular localization, selection of interacting partners, enzymatic functions and/or stability (Ashibe et al., 2007). In that context, while initial biochemical analysis suggested that all the variants of SG2NA are localized in both cytosolic as well as in membrane fractions, they differ in their tissue profiles as well as the expression levels in adult versus neonates. It is thus anticipated that different forms of SG2NA might have distinct roles in tissue differentiation and development. Two lines of evidences directed our interest towards investigating the potential role of variants of SG2NA in cell proliferation/growth. Firstly, it was initially isolated as a tumor antigen upregulated during S to G2 phase of cell cycle. Secondly, upon experimentation, we found that the 35 kDa form was more abundant in neonatal tissues and in immortalized cell lines (and additionally, the 35 kDa variant was found to be similar to an EST clone derived from mammary tumor). As expected, except the 83 kDa, its three other variants i.e., 87, 78 and 35 kDa were upregulated upon serum stimulation (and downregulated by serum starvation) with subtle differences in their kinetic (sustained versus transient induction). Therefore, these isoforms might play distinct roles while cells switch from quiescent to growing mode and vice-versa.

Finally, a comprehensive analysis of the splicing variation of SG2NA in various species was not an objective of this study. Nevertheless, we had examined its profile in embryonic chicken and observed a conserved pattern of splice variation (heart versus brain) as well. Though this study did not reveal the function of SG2NA, it nonetheless exemplified the importance of this regulatory molecule.

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